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Carbon nanotubes-based chemiresistive biosensors for detection of microorganisms

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ABSTRACT

The paper reports carbon nanotube (CNT)-based immunosensors for the detection of two types of microorganisms, bacteria and viruses. The pathogen *Escherichia coli* O157:H7 and the bacteriophage T7 were selected as model for bacteria and viruses, respectively, while *E. coli* K12 and the bacteriophage MS2 were used to assess the selectivity of the biosensor. The transduction element consisted of single-walled carbon nanotubes aligned in parallel bridging two gold electrodes to function as a chemiresistive biosensor. Single-walled carbon nanotubes (SWNTs) were functionalized with specific antibodies (Ab) for the different microorganisms by covalent immobilization to the non-covalently bound 1-pyrene butanoic acid succinimidyl ester. A significant increase in the resistance of the device was observed when the biosensor was exposed to *E. coli* O157:H7 whole cells or lysates with a limit of detection of 10^5 and 10^3 CFU (colony forming units)/mL, corresponding to 10^3 and 10^1 CFU/chip, respectively, while no response was observed when the biosensor was exposed to the *E. coli* K12. In the case of virus detection, a significant resistance increase was detected due to interaction of the bacteriophages with the Abs, with a limit of detection of 10^3 PFU/mL corresponding to 10^1 PFU/chip and excellent selectivity against MS2 bacteriophage. The sensor exhibited a fast response time of ~5 min in the case of bacteriophage detection, while the response time for the detection of bacteria was 60 min.

1. Introduction

Detection of pathogens by the traditional culture dependent methods is often tedious and time consuming, which has prompted the search for faster assays such as ELISA or PCR-based detection systems. Recently, real-time PCR has revolutionized the diagnosis of microorganisms in clinical laboratories (see Espy et al., 2006 for review). However, depending on the sample matrix, nucleic acids extraction can be a challenging step. Moreover, this method requires trained personnel and is still expensive, and therefore cannot be afforded by all laboratories. To overcome these limitations, the advances in micro- and nano-electronics have prompted the development of new class of biosensor formats for detection of pathogenic microorganisms. For instance, different biosensor formats have been reported for the detection of *Escherichia coli*, including optical biosensors such as integrating waveguide biosensor (Zhu et al., 2005), photodiode based detection (Song and Kwon, 2009) or electrochemical detection systems (Liao et al., 2007; Zhang et al., 2009), with very good limit of detection. However, the majority of these assays require the use of labels for the detection and therefore a more straightforward methodology is needed. In the case of virus detection, electrochemical impedance spectroscopy or surface plasmon resonance assays have proven successful for the detection of bacteriophages (i.e. viruses that infect bacteria) (Munoz-Berbel et al., 2008; Garcia-Aljaro et al., 2008, 2009). However, the fabrication of these biosensors is cumbersome and/or the limit of detection is significantly lower than the desired.

Since their discovery in 1991 (Iijima, 1991), carbon nanotubes (CNTs) have attracted the interest of researchers belonging to different disciplines throughout the world. Their unique physical and chemical properties make them very promising materials for their use in nanotechnology, which is reflected by the increasing number of studies published in the last decade. CNTs have a tubular structure composed by monomers of graphene that can be disposed in either a single sheet (single-walled carbon nanotube, SWNT) or in

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multiple sheets of graphene concentrically grown (multi-walled carbon nanotube, MWNT), which typically possess a diameter of few nanometers, displaying a one dimension structure with a high surface area-to-volume ratio and providing enhanced signal-to-noise ratio than the planar structures (see Merkoci et al., 2005; Trojanowicz, 2006, for review). Theoretically, this property is translated into a lower number of target molecules that are necessary to elicit a response to be detected by the biosensor.

SWNTs have proven useful for the construction of analytical devices for the detection of a variety of biomolecules, ranging from glucose, nucleic acids, small proteins, to even prokaryotic or eukaryotic cells (Allen et al., 2007; Lin et al., 2004; Wang et al., 2004; Maehashi et al., 2007; Villamizar et al., 2008, 2009). Most of the fabricated devices have been used as field-effect transistors (FETs) in which the change in the electrical conductance upon the binding of analyte molecule to the recognition molecule, or in case of gas sensing to the SWNT itself (Trojanowicz, 2006), is monitored. In the work of So and co-workers (So et al., 2008) a biosensor for the detection of *E. coli* whole cells was reported using an aptamer-functionalized SWNTS biosensor in which 2 out of 3 chips gave a positive response at a concentration of 10^4 CFU/mL *E. coli*. In this kind of sensors the mechanism of sensing is thought to occur through the Schottky barrier modulation effect as well as the chemical gating effect, although this issue is still unresolved (Heinze et al., 2002; He et al., 2009). Single SWNT or a network of SWNTs, grown using chemical vapor deposition of Si/SiO_2 followed by patterning of source and drain electrodes, are common configurations methods used by researchers working in nanosensors (Villamizar et al., 2009; Allen et al., 2007). Although direct growth offers good control on positioning, it requires specialized materials and patterning techniques. Recently, a simpler method involving drop casting or SWNT suspension with or without dielectrophoresis on prefabricated microelectrodes was introduced (Lim et al., 2010; Cella et al., 2010) allowing wider use of SWNTs-based FET platform.

In this study, we have developed a simple chemiresistive-based sensing platform for the detection of two types of microorganisms, bacteria and viruses, based on a network of parallel aligned SWNTs bridging two gold electrodes acting as source and drain as the transducer element. The specificity of the biosensor relies on the specific capture of the target microorganisms by functionalization of the biosensor with specific antibodies (Abs). To this end, SWNT were functionalized through covalent binding of the Ab to 1-pyrene butanoic acid succinimidyl ester (PBASE), previously adsorbed onto the SWNTs. The performance of this biosensor for the detection of the different microorganisms was evaluated with the pathogen *E. coli* O157:H7, as model for bacteria, and the bacteriophage T7, as model for viruses, while the *E. coli* K12 strain and the bacteriophage MS2 were used as negative controls to evaluate the biosensor selectivity.

2. Experimental

2.1. Bacterial strains and bacteriophages

Heat killed *E. coli* O157:H7, purchased from KPL Inc. (Gaithersburg, MD, USA) at a concentration of 10^9 CFU/mL, were used in this study. The heat treatment did not affect the recognition of the strain by the anti-*E. coli* O157:H7 antibodies (Abs) as confirmed with ELISA tests.

E. coli K12, a laboratory strain, was used to evaluate the biosensor selectivity. A stock culture of *E. coli* K12 was prepared by growing the cells overnight in Luria Bertani (LB) broth at 37 °C with continuous agitation, at the end of which the cells were harvested by centrifugation and washed three times in phosphate buffer (PB). Ten-fold serial dilutions of the stock culture were prepared in sterile PB and 0.1 mL of each were plated on LB agar, and the number of colonies after overnight incubation at 37 °C was counted for quantification.

A bacteriophage T7 (ATCC BAA-1025-B2) stock was prepared using *E. coli* (ATCC BAA-1025) as bacteriophage host. Phage concentration was determined by the double layer agar method (Adams, 1959). A bacteriophage stock of 10^9 PFU/mL was 10-fold serially diluted in PBS for assessment with the fabricated biosensors. MS2 phage was used as negative control in the selectivity studies. A stock of this phage was prepared using *E. coli* HS(pFamp)R as host strain for phage replication and the concentration was determined similarly as described above for the T7 phage.

2.2. Preparation of bacterial lysates

The bacterial cells were sonicated for 5 min with pulses of 15 s using a Virtis sonicator (Virsonic 600). Lysis of the cells was confirmed under optical microscope. The cell lysates were used immediately.

2.3. Biomolecules and chemical reagents

Anti-*E. coli* O157:H7 monoclonal antibodies (Abs) was purchased from Remel (Lenexa, KS, USA). Anti-T7 monoclonal Abs was purchased from (Millipore, MA, USA). All the chemicals used were of analytical grade and were used as received without any further purification unless otherwise stated. The PB stock solution (0.1 M, pH 7.4) was prepared by mixing 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 until the desired pH. A 10-fold dilution of this stock was used as working solution. All the solutions were prepared in double distilled water unless otherwise stated.

2.4. Microelectrode fabrication

Gold microelectrodes were used as the base sensing platform for the fabrication of the biosensor. The electrodes were prepared by first deposition of a chromium adhesion layer of 200 Å on a 100 oriented p-doped silicon wafer with 300 nm SiO_2 layer, followed by deposition of a 1800 Å thick gold contact layer. The resulting microelectrodes were separated by a 3 μm and 5 μm gap for bacteriophage and bacteria detection, respectively. The microelectrodes were cleaned in acetone (analytical grade, Fischer Scientific, Fair Lawn, NJ, USA) by sonication in water bath for 15 min, followed by an additional 15 min sonication in double distilled water. The microelectrodes were then dried with N_2 and either used immediately or stored under vacuum in a desiccator.

2.5. Biosensor fabrication

A SWNT suspension was prepared by suspending 0.02 mg/mL CNTs in dimethylformamide (DMF) (Fisher Scientific, Fair Lawn, NJ, USA) followed by 90 min sonication in a water bath. The solution was centrifuged at 15,000 rpm for 3 h and the supernatant was collected. The SWNT suspension prepared this way can be kept for at least 1 month, although a 60 min sonication step is necessary before each use.

The biosensor fabrication involved three steps: alignment, annealing and functionalization of the SWNTs. Alignment of SWNTs was performed by depositing a drop of 0.4 μL SWNT suspension in the microelectrode gap, and applying AC electrophoresis at 4 MHz for few seconds until a resistance of around 1–10 MΩ was obtained. The aligned SWNTs were then annealed by incubation of the microelectrodes for 1 h at 300 °C in a mixture of N_2:H_2 (95:5, v/v) to obtain a good contact between the gold and the SWNTs and remove any DMF residues. The third step included functionalization of the device by the corresponding Ab. Briefly, SWNTs were incubated in a solution containing 6 mM 1-pyrene butanoic acid succinimidyl

ester (PBASE) (Invitrogen, Carlsbad, CA, US) diluted in DMF for 1 h in darkness. The nanotubes were then rinsed with DMF, followed by PB, and incubated overnight at 4 °C with 50 μL of the corresponding Ab stock solution. After washing with PB, the biosensor was incubated for 1 h with 0.1 M ethanolamine, followed by 1 h with 0.1% Tween 20 to quench the unreacted succinimidyl ester and passivating uncoated SWNT, respectively. The sensor was checked for stability and then incubated for different periods with increasing bacterial or bacteriophage concentrations. In order to confirm that the observed change in resistance was due to the interaction of the microorganism with the specific Ab, a control for each functionalization procedure was performed, where no Abs were incorporated. Instead, functionalization was performed with BSA (0.5 mg/mL) (Sigma–Aldrich). The selectivity of the biosensor was assessed by incubation of the biosensor with the *E. coli* K12 strain, and the bacteriophage MS2, which are not recognized by the Abs.

2.6. Electrical measurements

Linear sweep voltammetry measurements were performed using a semiconductor parameter analyzer (Model 4155A, Agilent Technologies Inc., CA, USA) from −0.2 V to 0.2 V. The slope of the current/voltage (I/V) curves between −0.1 V and 0.1 V for each concentration was calculated using linear regression analysis, and the inverse of this value, the resistance (R), was calculated (Fig. 1). All measurements were carried out in 10 mM PB (pH 7.4), previously filtered with a 0.22 μm-pore size filter (Millipore, MA, USA). The results are expressed as a mean of three replicates with its corresponding standard deviation.

3. Results and discussion

3.1. Bacteria detection

A biosensor for the detection of bacteria using the pathogen *E. coli* O157:H7 as a model was constructed and evaluated in terms of response time, sensitivity, and specificity/selectivity. In this case, the transducer element of the sensing platform was parallel oriented SWNTs bridging a 5 μm gap between two gold electrodes and functionalized with PBASE followed by covalent binding of monoclonal anti-*E. coli* O157:H7 Abs. A scheme of the developed biosensor and an example of the current–voltage dependent response of the biosensor after each functionalization step is provided in Fig. 1. The current decrease, i.e. resistance increase, is attributed to accumulation of negative charge from antibodies, bacteria reducing the hole carriers of p-type semiconductor SWNTs and/or scattering potential and Schottky barrier modulation.

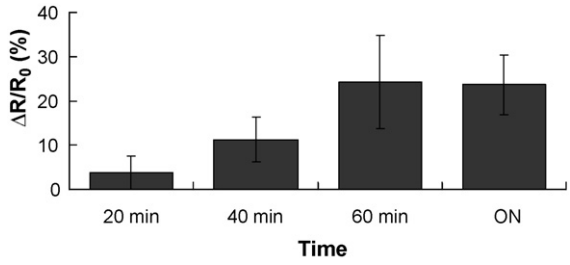

Fig. 2. Time-dependent response of the CNT-biosensor after exposure to *E. coli* O157:H7 whole cells at a concentration of 10^7 CFU/mL; ON (overnight incubation).

The initial investigation was focused on determining the time-dependent response of the biosensor to establish the required incubation time for maximum sensor response. To this end, the biosensor was incubated with 10^7 CFU/mL of *E. coli* O157:H7 at room temperature and the biosensor response was measured as the normalized increase in resistance, $\Delta R/R_0$ [$\Delta R = (R - R_0)$, R is the resistance of the biosensor after exposure to sample and R_0 is the initial biosensor resistance, after Ab immobilization, ethanolamine and Tween 20 blocking and washing in buffer], as a function of incubation time. As observed in Fig. 2, the biosensor response increased with incubation time up to 40 min attaining a plateau value of 24% with respect to initial sensor resistance after 1 h incubation. Accordingly, a 60 min incubation time was used in the subsequent experiments.

Fig. 3a shows the calibration plot for the *E. coli* O157:H7 immunosensor. As shown in the figure, the biosensor response was a linear function of logarithm of the bacterial concentrations between 10^3 CFU/mL and 10^7 CFU/mL ($r^2 = 0.93$). The limit of detection was 10^5 CFU/mL, which equates a total of 10^3 CFU/chip. Atomic force microscopy revealed attachment of bacteria onto SWNTs (Fig. 4). Previous studies have shown that SWNTs functionalized with specific Abs are able to capture bacteria (Elkin et al., 2005; Villamizar et al., 2008, 2009). However, in order to confirm that the observed resistance increase was the result of specific Ab-bacteria interactions and not non-specific binding of the *E. coli* O157:H7 to the SWNTs, a control experiment was performed in which the response of SWNTs functionalized with BSA instead of Ab was investigated. No significant resistance increase was observed

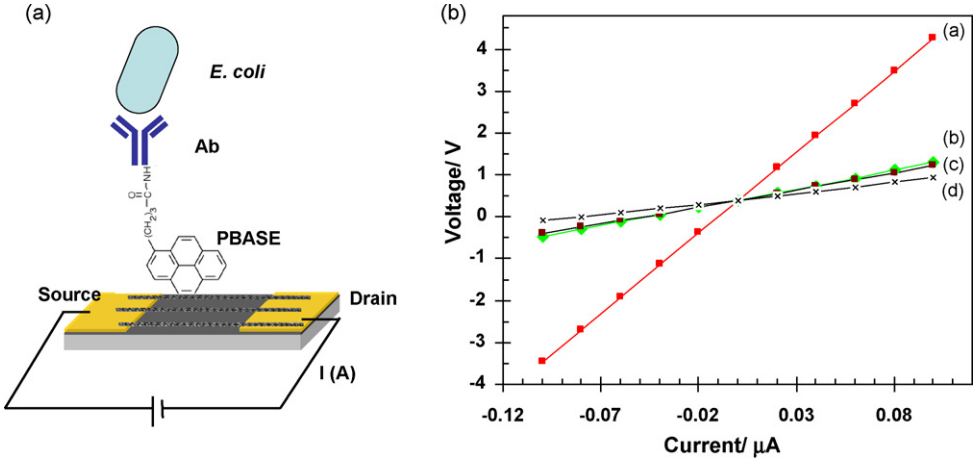

Fig. 1. Schematic representation of the CNT-biosensor used for *E. coli* detection (a) and typical current changes observed after each functionalization step (b). A decrease in the current was observed after fuctionalization of the CNTs with (a) PBASE (1-pyrene butanoic acid succinimidyl ester) followed by (b) coupling of antibody (Ab) to PBASE. Blocking of the biosensor with ethanolamine (c) was used to prevent unspecific binding onto the CNTs causing a slight decrease in the current. In the example capture of *E. coli* O157 is shown (d).

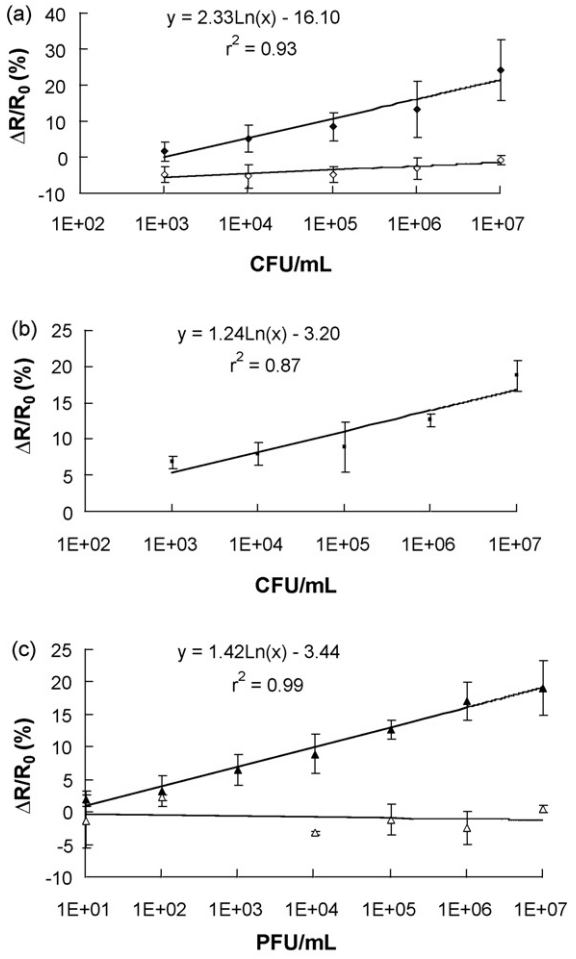

Fig. 3. Calibration plot of the response of the different CNT-biosensors. (a) *E. coli* O157:H7 biosensor functionalized with anti-*E. coli* O157:H7 Abs subjected to increasing concentrations of *E. coli* O157:H7 whole cells (♦), and response of a BSA functionalized device used as negative control (◊); (b) *E. coli* O157:H7 biosensor functionalized with anti-*E. coli* O157:H7 Abs subjected to increasing concentrations of *E. coli* O157:H7 cell lysates; (c) T7 phage CNT-biosensor functionalized with anti-T7 Abs to increasing concentrations of the T7 bacteriophage (▲), and response of a BSA functionalized device used as negative control (△).

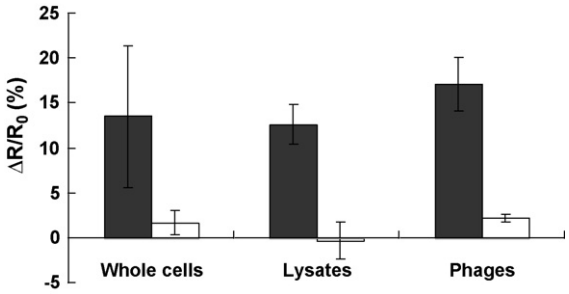

Fig. 5. Analysis of the selectivity of the developed biosensors. The biosensors were exposed at a concentration of 10^6 CFU/mL or 10^6 PFU/mL of bacteria and bacteriophages, respectively. In the case of the *E. coli* O157 biosensor the positive control (black box) consisted of *E. coli* O157:H7 whole cells or cell lysates, while the negative controls (white box) consisted of the *E. coli* K12 strain that does not possess the O157 antigen. In the case of the bacteriophage biosensor, the positive control (black box) consisted of the T7 phage, while the negative control (white box) consisted of the MS2 phage.

(−0.5% ± 1.27), suggesting that there was no non-specific binding of *E. coli* O157 to the SWNTs.

To investigate the biosensor selectivity, the sensor response to 10^6 CFU/mL of a related strain, *E. coli* K12, which does not display the O157 serotype, was measured. As expected, and according to previous ELISA tests (data not shown), exposure to *E. coli* K12 cells produced a response of 1.75% ± 1.41, which is 8-fold lower than the sensor response to 10^6 CFU/mL of *E. coli* O157:H7 (Fig. 5).

Gram-negative bacteria such as *E. coli* have a theoretically overall negative charge due to the charges present in their external membrane in the lipopolysaccharide, were the O antigen resides. Their attachment onto SWNTs is expected to cause an increase in the resistance on p-type SWNTs in the Debye length where the biosensor is sensitive (Chen et al., 2004). In this work, the sensing platform consisted of a molecule of few nanometers (PBASE is around 2–3 nm, Prakash et al., 2006) attached to the SWNT followed by attachment of a monoclonal Ab that is typically around 4 nm size. Because these bacteria have a size of around 0.8 μm × 1.5 μm, it is expected that only the charges in the Debye length will produce some effect in the biosensor.

In an attempt to improve the limit of detection of the biosensor, detection of lysed cells was explored. Using lysed cells improved the limit of detection of the immunosensor to an equivalent concentration of 10^3 CFU/mL, corresponding to 10^1 CFU/chip (Fig. 3b).

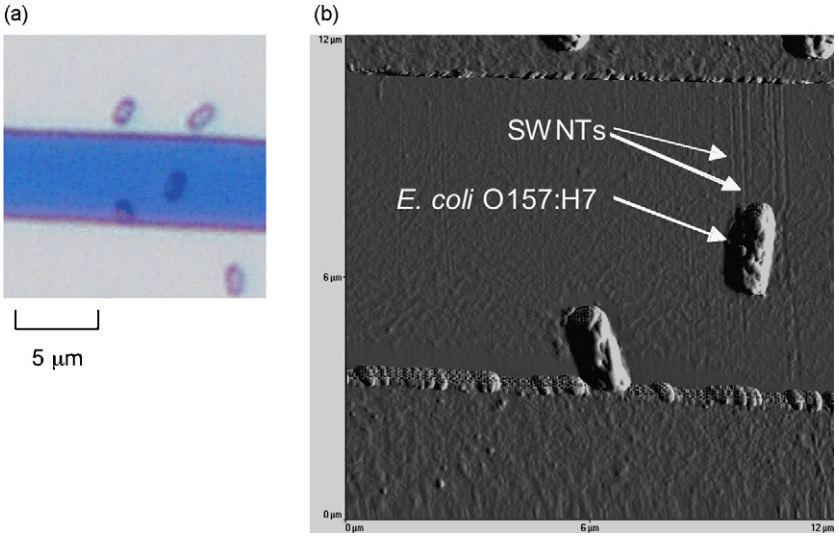

Fig. 4. Optical microscope image (a) and AFM image (b) showing the functionalized biosensor after exposure to *E. coli* O157:H7 whole cells.

The plausible explanations for the improved limit of detection with the cell lysates could be 1) higher diffusivity of the lysates compared to whole cells because of particle size, improving the accessibility of the cells O157:H7 antigen to the Abs immobilized on SWNTs as suggested by (Upadhyayula et al., 2008) and 2) reduced steric hindrance. An additional advantage of detecting lysate over whole cells is significantly lower standard deviation, i.e. improved reproducibility, which is attributed once again improved interaction between the antigen and antibody because of smaller particle size and a higher diffusivity of smaller particles.

With regards to the control, once again a significantly smaller response of $-0.25\% \pm 2.05$ was observed for the same concentration of lysed *E. coli* K12 cells compared to *E. coli* O157:H7 (Fig. 5), confirming excellent selectivity of the immunosensor.

3.2. Viruses detection

The application of the SWNT-based transducer platform was extended to the detection of bacteriophage T7 as model for virus using anti-T7 functionalized SWNTs. The biosensor was characterized in terms of sensitivity, dynamic range and limit of detection for the T7 bacteriophage. The selectivity was evaluated against the bacteriophage MS2.

The biosensor showed a linear response for a bacteriophage T7 concentration between 10^2 PFU/mL and 10^7 PFU/mL corresponding to 1–10^5 PFU/chip, with a maximum response of 19% at a concentration of 10^7 PFU/mL (Fig. 3c), with an incubation time of only 5 min. In comparison, a negligible response was observed for the same concentration of MS2 bacteriophage (Fig. 5) as well as with BSA functionalized SWNTs ($-1.19\% \pm 2.49$).

Bacteriophages can be regarded as negatively charged particles (Clifton and Madison, 1931) and therefore their sensing with SWNTs is expected to behave similarly to the sensing of bacteria as stated above. However, the performance of the biosensor was better in the case of bacteriophage sensing compared to bacteria sensing probably due to the fact that bacteriophage T7 has an icosahedral head of around 45 nm diameter with a very short tail, which is much smaller than an *E. coli* O157. The spherical shape and smaller size of bacteriophages compared to bacteria are expected to provide a better diffusion of them in the PB solution towards the attached Abs as in the case of cell lysates. The different shape and size of bacteriophage with respect to the bacteria is expected to affect also the response time of the biosensor, since bacteriophage T7 is more than time times smaller than an *E. coli* O157:H7 and therefore should diffuse faster.

Irrespective, physisorption of the Abs onto the gold cannot be discarded, which could contribute to the response through the Schottky barrier modulation effect at the nanotube-gold contact (Lee et al., 2008). In fact, some reports have shown that adsorption of proteins onto the metal-nanotube contacts constitute a more significant contribution to the biosensing signal than adsorption of the proteins along the SWNTs itself (Heinze et al., 2002; Chen et al., 2004; Byon and Choi, 2006). Thus contribution of the Schottky barrier modulation effect to the response of the biosensor could also be favored in the case of bacteriophages and cell lysates because of their smaller size, making them more accessible to the SWNT–gold junction.

4. Conclusions

To summarize, we have developed highly sensitive and selective SWNTs-based chemiresistive biosensors for detection of human pathogen *E. coli* O157:H7 and bacteriophage T7 using a facile and affordable technology that can be employed in most laboratories. A limit of detection of 10^3 CFU/mL and 10^3 PFU/mL was achieved for detection of *E. coli* O157:H7 lysed cells, and the bacteriophage T7, respectively. Although lower limit of detection has been reported for bacteria and bacteriophages using other techniques, the advantages of this biosensor rely on its simple measurement performance, allowing measurements to be performed almost in a real-time manner even at room temperature. The integration of nanostructures such as SWNTs into microelectronic devices in combination with the use of specific recognition molecules such as Abs has shown promising results for the next generation of truly miniaturized, sensitive, and cost-effective biosensors. Incorporation of microfluidic technology is expected to lower the limit of detection of these devices and reproducibility. Since the sensing area of our device is of few micrometers in size, recirculation of the sample may increase the chance for a single particle to reach this area.

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